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(71) Applicant: CELLCO ADVANCED BIOREACTORS, INC. [US/US]; 5516 Nicholson Lane, Kensington, MD 20895 (US).

(72) Inventors: KNAZEK, Richard, A. ; 9424 Locust Hill Road, Bethesda, MD 20814 (US). KIDWELL, William, R. ; 10905 Lowell Court, Ijamsville, MD 21254 (US).

(74) Agents: STERN, Marvin, R. et al.; Fleit, Jacobson, Cohn, Price, Holman & Stern, The Jenifer Building, 400 Seventh Street, N.W., Washington, DC 20004 (US).

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(57) Abstract

This invention provides improved methods for *in vitro* culturing of therapeutic quantities of expanded subpopulations of lymphoid cells for use in methods of adoptive immunotherapy. Lymphoid cells, which are derived from a patient, are grown in a hollow cell fiber bioreactor culture system in the presence of at least one growth promoting substance, that specifically expands subpopulations of the lymphoid cells that are useful in methods of adoptive immunotherapy, including the treatment of cancer and genetic disorders. The improved methods of this invention provide convenient and inexpensive means for generating the large numbers of biologically active cells that are needed for adoptive immunotherapy.

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METHOD FOR THE PRODUCTION OF IN VITRO EXPANDED LYMPHOID CELLS FOR USE IN ADOPTIVE IMMUNOTHERAPY

This invention is a continuation-in-part of U.S. application serial number 07/238,445, which was filed on August 31, 1988, the subject matter of which is incorporated herein by reference thereto.

BACKGROUND OF INVENTION

A recent and highly promising development in the therapeutic treatment of diseases involves the use of adoptive immunotherapy (See, e.g. Belldegrun et al. (1989) Chapter 12, in Urologic Oncology, Lepor et al. (eds.), Kluwer Academic Publishers, Boston). Adoptive immunotherapy is the passive transfer to an afflicted host of in vitro expanded lymphoid cells that are therapeutically effective in treating the disease via destruction of the affected cells of the host by virtue of specific interaction with the host's affected cells or by furnishing therapeutically effective substances to the host.

Adoptive immunotherapy involves the isolation of lymphoid cells from an afflicted individual, the selective expansion of subpopulations of lymphoid cells, which have a high degree of reactivity or that have been modified to have a high degree of reactivity against cancerous or other disease affected cells, in vitro in the presence of growth promoting substances, such as the interleukins, that mediate the expansion of reactive subpopulations of the cells populations and the reintroduction of large numbers (1×10^{10} to 5×10^{11}) of the expanded cells into the afflicted host, whereby the in vitro expanded lymphoid cells destroy the cancerous

or otherwise affected cells or furnish therapeutically effective substances. For some methods of adoptive immunotherapy the lymphoid cells are expanded in the presence of target antigens or cells exhibiting such target antigens as well as growth promoting substances. The cells exhibiting such target antigens are generally derived from the afflicted individual. Target antigens can be isolated from the cells of an afflicted individual or may be prepared by synthetic means, such as by recombinant DNA technology or peptide synthesis. The use of adoptive immunotherapy for the treatment of cancer has been studied extensively. (See, e.g., Belldegrun et al., supra.). Adoptive immunotherapy has been used to treat cancers of the kidney, colon, lungs, renal and breast, melanomas, lymphomas, and sarcomas (see, e.g., Belldegrun et al., supra., Topalian et al. (1988) J. Clin. Oncol. 5: 839-853, Rosenberg (1987a) U.S. Patent No. 4,690,915, which disclosure is herein incorporated in its entirety by reference thereto; see, also, Rosenberg, et al.(1987b) New Eng. J. Med. 316: 889-897, Rosenberg (1986a) at pp. 55-91 in Important Advances in Oncology, DeVita et al. (eds.), JB Lippincott, New York), Yron et al. (1980) J. Immunol. 125:238, and Rosenberg (1985) Cancer 55: 1327).

Adoptive immunotherapy can also be used for the treatment of other diseases, including viral diseases and genetic defects. For example, lymphoid cells can be isolated from a patient, modified by the incorporation of cloned DNA into the DNA of the lymphoid cells, to encode an enzyme that corrects a genetic defect or to encode a therapeutically effective agent, cultured and then reintroduced into the patient in whom the cloned DNA is

expressed. Immunoreactive lymphoid cells that have been cultured in the presence of specific viral target antigens and specific factors, such as IL-2, may be useful for the treatment or immunization of individuals against such virus.

As discussed above, some methods of adoptive immunotherapy require the use of expanded subpopulations of lymphoid cells that are produced from lymphoid cells when the lymphoid cells have been cultured in the presence of specific antigens, such viral antigens or the antigenic portions of the immunologically active viral proteins or antigens, or in the presence of cells bearing such antigens, such as tumor cells or virally infected cells. The antigen that is used may be that which is presented on a cell surface, such as an irradiated tumor cell, it may be a purified antigen, or may be it may be synthetic antigen, produced by methods such as by the expression of cloned DNA or peptide synthesis. If the particular antigenic source is tumor cells, then the cells that are produced when the tumorous cells are cultured in the presence of IL-2 are tumor infiltrating lymphocytes (herein after TILs).

The mediators that are necessary for expansion of such tumor specific lymphoid cells are growth promoting substances and include mitogens, cytokines and lymphokines. Mitogens are responsible for antigen independent development of lymphoid cells, cytokines are factors, such as lymphokines or monokines, that are produced by cells that affect other cells, and lymphokines are substances that are produced and secreted by activated T lymphocytes and that affect other cell types. In particular, it is known that certain

lymphokines, such as IL-2, mediate specific expansion of subpopulations of lymphoid cells that bear specific phenotypic surface markers and that specifically recognize certain antigens on the surfaces of affected cells. The lymphokine, interleukin-2, hereinafter IL-2, has been used to expand certain populations of lymphocytes that have a high degree of antitumor reactivity (see, e.g., Rosenberg (1987a), supra., see, also, Rosenberg (1987b), Rosenberg (1986a), supra., Yron et al., supra., Rosenberg (1985), supra.,).

IL-2, which was originally identified as a T cell growth factor, has been used to generate certain lymphoid cells that possess antitumor reactivity against the syngeneic or allogeneic tumor bearing host. For example, incubation of resting lymphocytes, which are obtained from tumor bearing hosts, including human and murine hosts, in IL-2 for three to four days results in the expansion of subpopulations of lymphocytes that are capable of lysing natural killer cell (hereinafter NK)-resistant tumor cells, but not normal cells (see, e.g., Belldegrun et al., supra.). This phenomenon is called lymphokine activated killing (hereinafter LAK) and the lymphocytes that are responsible for this phenomenon consist of two types of cells. The first type of cells is called LAK cells and the second type of cells is called TILs.

LAK cells can be obtained from both normal individuals and from individuals afflicted with cancer or other diseases. LAK cells appear to constitute a lytic system of cells that is distinct from NK and cytolytic T lymphocytes (hereinafter CTL) cells. LAK cell precursor and effector cells possess phenotypes that are typical of

NK cells (see, e.g., Phillips et al., (1986) J Exp. Med. 164: 814), but have the ability to lyse fresh, noncultured NK-resistant allogeneic primary or metastatic cancer cells, and can be generated from peripheral blood lymphocytes (hereinafter PBL), thymus, spleen, lymph node, bone marrow and thoracic duct cells (see, e.g., Belldegrun et al., supra., at pp. 215-216). LAK cells also have the ability to lyse fresh autologous and allogeneic tumor cells and many cultured cell lines. Whereas LAK precursors have neither T nor B cell surface markers, LAK cells appear to be Thy 1.2-positive and Ia-negative and the majority of the cytotoxic activity resides in the FcR-positive subpopulation (see, e.g., Lefor et al. (1989) at pp. 39-56 in Functions of the Natural Immune System, Reynolds et al., eds., Plenum Pub. Corp.). LAK cells have also been shown to mediate antibody-dependent cellular cytotoxicity (ADCC) (see, e.g., Lefor et al., supra.). It has been demonstrated that IL-1 and tumor necrosis factor (TNF) increase such IL-2 induced ADCC activity and that such increase is associated with a correlative increase in the lytic potential of lymphoid cells that have been so induced (see, e.g., Eisenthal et al. (1989) J. of Immunol. 142: 2307-2313).

TIL cells are lymphocytes that infiltrate into tumors, against which a host's immune system is mounting an immunological response, and can be isolated therefrom (see, e.g., Yron et al., supra. and Anderson et al., supra.). TIL cells are found to have greater specificity than LAK cells for autologous cells and greater efficacy than LAK cells in adoptive immunotherapy of cancer (see, e.g., Yron et al., supra.). TIL cells

have been obtained from resected human tumors, including cancers of the kidney, colon, and breast, melanomas, and sarcomas.

In vitro incubation of cells that have been obtained from a tumor and grown in the presence of IL-2 results in the expansion of activated T cells within the tumor and the destruction of tumor cells or tissue. After 2-3 weeks of culture, the tumor cells have all been destroyed and the culture consists of lymphoid cells that have the phenotype of cytolytic T lymphocytes (CTL) (see, e.g., Muul et al. (1987a) J. Immunol 138: 989, Topalian et al., supra. and Itoh, et al. (1986) Cancer Res. 46: 3011). Some human TIL cells exhibit a high specificity for their autologous tumors.

TIL cells also show promise for use in methods of genetic therapy (see, e.g. Culliton (1989), "News and Comment" in Science 244: 1430-1433.) They provide a source of autologous cells that can be modified by the insertions of DNA encoding a desired protein, cultured, and reintroduced into the patient. The desired protein may be a therapeutically effective protein, such as tumor necrosis factor, which is used in cancer therapy, CD4 receptor to which HIV binds, an enzyme, for which the treated host is deficient, or a it may be a marker protein, whereby the fate of the TIL cells in the treated host may be studied.

In addition to LAK and TIL cells other types of lymphoid cells have also been identified as possessing antitumor reactivity. For example, RLN (regional draining lymph node) cells are a population of lymphoid cells, which have antitumor reactivity, that are derived from the regional draining lymph nodes of tumor bearing

mice that have been immunized with weakly immunogenic tumors (Stephenson et al. (1989) Surgery 105: 523-528). RLN cells are therapeutic effector cells and represent a different cell population than LAK cells.

Because many cancer patients do not respond to adoptive immunotherapy, studies are underway to identify other lymphokines, cytokines, and/or mitogens that may be useful alone or in combination with Il-2 in expanding subpopulations of lymphoid cells for use as adoptive immunotherapeutic agents. Although IL-2 has primarily been used to generate such subpopulations of lymphoid cells, other lymphokines, such as IL-4, IL-6 and other interferons, and TNF have also been shown to be to be useful in the production of in vitro expanded lymphoid cells and may also prove to be useful in expanding specific subpopulations of lymphoid cells. For example, IL-4 (also called BSF-1) is a glycoprotein that is derived from T cells and has been shown to induce LAK activity if the lymphoid cells are first stimulated with IL-2, but is inhibitory if the cells are not pre-stimulated (Kawakami et al. (1989) J. of Immunol. 142: 3452-3461) IL-4 also has been shown to be capable of stimulating the growth of TIL cells both alone and in conjunction with IL-2. IL-4 appears to enhance the growth of TIL cells and concomitantly inhibit the growth of $NKHI^{+}$ cells, which are responsible for non-specific killer activity (Lotze et al. (1989) at pp. 167-179 in Human Tumor Antigens and Specific Tumor Therapy, Alan R. Liss, Inc., see, also, Kawakami et al., (1988) J. of Exptal. Med. 168: 2183-2191.).

The possibilities for uses of adoptive immunotherapy are almost limitless. Not only can it be

used for the treatment of cancer by specific interactions between the lymphoid cells and the tumor, but, as discussed above, it can be used for the treatment of genetic diseases and as a means of delivering antitumor agents and other therapeutic agents. Lymphoid cells can be removed from an individual who is suffering from a genetic disease that results from an enzyme or hormone deficiency. A wild type copy of the gene of interest can be inserted into the lymphocytes and the lymphocytes can be cultured in the presence of selective agents and reintroduced into the afflicted individual. Alternatively, the lymphoid cells can be genetically modified to express a therapeutically effective anticancer or antiviral agent, such as interferon or TNF, and then reintroduced into the patient (See, e.g., Genetic Eng. News. Vol. 9, No. 3, March 1989 and p. 133 in Business Week/May 1, 1989).

In practicing adoptive immunotherapy it is, however, necessary to develop methods not only for the identification of therapeutically useful subpopulations of lymphoid cells, but to develop methods for the generation of large quantities of such cells. Adoptive immunotherapy of human cancers and other disorders is a highly promising treatment, but the inability to generate clinically useful numbers of immunoreactive lymphoid cells has been a major obstacle to the use of adoptive immunotherapy (see, e.g. Rosenberg et al. (1986b) Science 233: 1318-1321 and Culliton ,supra.). There is, thus, a need for methods for the large scale production of lymphoid cells and for methods that can be readily adapted as new cells and agents for their expansion are identified.

Currently, the generation of sufficient numbers of expanded subpopulations of lymphoid cells, such as TILs, for administration to patients having metastatic disease is time consuming, inefficient, and prohibitively expensive. It is accomplished by growing TILs, which are derived from a metastatic lesion, in plastic, gas permeable culture bags, each of which holds about 1.5 liters of tissue culture medium that contains human serum albumin and recombinant human IL-2 (see, e.g., Muul et al. (1987b) J. Immunol. Meth. 101: 171-181, see. also Culliton, supra.) The cells grow and divide until they reach a maximum density of, at most, 2-3 x 10^9 cells per bag at which point they must be split into additional culture bags. It, thus, may require 50 to 150 bags to generate a sufficient number of cells a single clinical treatment of one patient. Over a 4 to 6 week time period the cells are grown and split into additional bags until a sufficient number of cells for clinical treatment are generated typically expanding into 100 to 150 bags, containing approximately 10^{11} cells. The cells, which are in a volume of 150 to 250 liters, must then be centrifuged and maintained under sterile conditions. The entire procedure, which generates enough cells for the treatment of a single patient, uses enormous quantities of mammalian cell culture medium, human serum albumin, and IL-2, as well as a great deal of time and manpower. Typically it requires about 5 hours to harvest the cells for a single treatment. Because this cell concentration method yields cells in dilute solution, is fraught with opportunities for contamination of the cultured cells, and is prohibitively expensive. Its use as a means to generate the therapeutically necessary quantities of

cells for clinical treatment is severely limited.

There is, thus, a need for the development of methods that can be used to efficiently and cost effectively grow the large numbers of biologically active *in vitro* expanded lymphoid cells that are suitable for use in methods of adoptive immunotherapy. Further, because of the wide range of disorders that can be treated by this method, there is a need to develop methods that can be readily adapted to changing protocols and to the many protocols for which such expanded populations of lymphoid cells will be used.

SUMMARY OF THE INVENTION

It is one object of this invention to provide an improved method for the large-scale production of *in vitro* expanded lymphoid cells that can be used in adoptive immunotherapy, comprising (a) inoculating the extra fiber space of a hollow fiber bioreactor with a suspension of lymphoid cells in a growth-promoting factor containing medium; (b) perfusing said bioreactor with tissue culture medium that contains an effective amount of at least one growth promoting substance that specifically expands a therapeutically useful subpopulation of said lymphoid cells, wherein said effective amount is an amount sufficient to effect said specific expansion, said tissue culture medium sustains the cell division and growth of said subpopulation, and said therapeutic use is adoptive immunotherapy; and (c) culturing said cells in said bioreactor in the presence of said growth promoting substance for a time sufficient to obtain a therapeutically effective number of said *in vitro* expanded lymphoid cells.

It is another object of this invention to provide an improved method for the production of in vitro expanded lymphoid cells for use in methods of adoptive immunotherapy, wherein said expanded lymphoid cells are TIL cells, comprising (a) suspending cells that are derived from a resected tumor tissue in cell tissue culture medium; (b) culturing said suspension in the presence of an effective amount at least one cytokine that is capable of promoting the expansion of tumor infiltrating lymphocytes, wherein said effective amount is a amount sufficient to effect the expansion of the tumor infiltrating lymphocytes in said suspension; (c) inoculating the extra fiber space of a hollow fiber bioreactor that is a component of a hollow fiber culture system with said cultured suspension of tumor infiltrating lymphocytes; (d) perfusing said bioreactor with tissue culture medium that contains an effective amount of at least one cytokine that is capable of promoting the expansion of tumor infiltrating lymphocytes, wherein said effective amount is an amount sufficient to effect said specific expansion and said tissue culture medium sustains the cell division and growth of said tumor infiltrating lymphocytes; and (e) culturing said tumor infiltrating lymphocytes in said bioreactor in the presence of said tissue culture medium for a time sufficient to obtain a therapeutically effective number of said tumor infiltrating lymphocytes.

It is another object of this invention to provide in vitro expanded lymphoid cells for use in methods of adoptive immunotherapy.

It is another object of this invention to provide in vitro expanded lymphoid cells for use in

methods of adoptive immunotherapy, wherein said expanded lymphoid cells are TIL cells.

It is another object of this invention to provide in vitro expanded lymphoid cells for use in methods of adoptive immunotherapy, wherein said expanded lymphoid cells are lymphoid cells whose genomes have been modified by the incorporation therein of cloned DNA.

It is another object of this invention to provide a method for preparing a conditioned medium for use in stimulating the growth of in vitro expanded lymphoid cells and as a source of biologically active growth promoting substances that specifically expand therapeutically useful in vitro expanded lymphoid cells, comprising removing the contents of the extra-fiber space of a bioreactor in which in vitro expanded lymphocytes have been cultured, pelleting and removing the cells from said contents of the extra fiber space to produce an extra fiber space cell supernatant; and dialyzing said extra fiber space cell supernatant against tissue culture medium to produce extra fiber space conditioned medium.

It is another object of this invention to produce conditioned medium for use in stimulating the growth of in vitro expanded lymphoid cells and as a source of biologically active growth promoting substances that specifically expand therapeutically useful in vitro expanded lymphoid cells.

It is another object of this invention to provide an improved method for producing in vitro expanded lymphoid cells, comprising culturing said cells in the presence of an effective amount of extra fiber space conditioned medium, wherein said amount is effective to stimulate the rate of growth of said

expanded cells at least about 50% more than the growth of said cells in its absence

This invention significantly improves the procedure for preparing therapeutically useful quantities of in vitro expanded lymphoid cells by providing an improved method for culturing said cells that can be adapted to the specific requirements of an adoptive immunotherapeutic procedure, whereby lymphoid cells are obtained from a patient, inoculated into a hollow fiber bioreactor culture system and cultured in the presence of an effective amount at least one growth promoting substance that specifically expands a therapeutically useful subpopulation of lymphoid cells.

In practicing this invention therapeutically useful yields of biologically active therapeutically effective in vitro expanded lymphoid cells are obtained using a convenient method that not only significantly reduces the costs associated with the production of such cells but significantly increases the numbers of cells that can be produced.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 presents a typical growth curve for TIL grown according to the methods of this invention. The TIL increase in cell number versus time elapsed from the date of excisional biopsy. Cells were obtained from the tumor that had been excised from patient W. The culture was initiated in gas permeable bags, when sufficient TIL had been grown in standard culture flasks from the enzymatically dispersed tumor, which contained the TIL, and then inoculated into the hollow fiber reactor on day 23 after excisional biopsy. -0-0-0- represents the growth curve of cells grown in gas permeable bags as

measured by an increase in cell number, which is plotted on the ordinate on the right side, versus duration of culture, which is plotted on the abscissa as days. After an initial lag period, the number of cells obtained from patient W (W-TIL) increased exponentially over time. Time = 0 indicates the date of excisional biopsy. TIL cells were withdrawn from one bag on day 23 and were inoculated into a CELLMAX™ hollow fiber bioreactor and harvested on day 44 (-▲-▲-▲-). Glucose consumption, as measured by a decrease in glucose concentration in the bioreactor perfusate, which is plotted on the ordinate on the left side, versus days in culture on the abscissa, increased logarithmically with time(-o-o-o-). + represents the replacement of the extra-fiber space (EFS) with fresh complete media. The hollow fiber inoculum of 4.3×10^8 cells on day 23 yielded 5.4×10^{10} cells at harvest.

Figure 2 depicts a growth curve showing G-TIL (TIL obtained from patient G) increasing in number versus time elapsed from the date of excisional biopsy. -0-0-0- represents the growth curve of cells grown in gas permeable bags as represented by an increase in cell number. After an initial lag period, the number of cells obtained from patient G increased exponentially over time measured from the date of excisional biopsy. TILs were withdrawn from one bag on day 16, inoculated into a CELLMAX™ hollow fiber bioreactor and harvested on day 30 (-▲-▲-▲-). Glucose consumption, as measured by a decrease in glucose concentration in the perfusate, which is plotted on the ordinate on the left side versus duration of culture, increased logarithmically with time(-o-o-o-). + represents the replacement of the EFS with fresh complete medium. The inoculum of 1.0×10^8 TIL

yielded a 1.5 x 10^{10} TIL harvest. Perfusion of the hollow fiber bioreactor was re-instituted on day 30, the day of the first harvest, and the residual TIL expanded again to 1.5 x 10^{10} for a second harvest on day 51. Perfusion was again re-instituted and the residual cells were once again permitted to expand for a third harvest of 2.1 x 10^{10} cells on day 73.

Figures 3 show scanning electron micrographs of TIL cells grown by the methods of this invention from patient W within the extra-fiber space of a CELLMAX™ hollow fiber bioreactor.

In Figure 3a the space between the large, ovoid hollow fibers is filed with a nearly solid mass of TIL. The ovoid shape of the normally cylindrical hollow fibers and the space between the TIL mass and the fiber surfaces are artifacts of histologic preparation.

Figure 3b shows a higher magnification so that the individual cells near the outer surface of a single hollow fiber can be seen.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All publications mentioned herein are incorporated by reference thereto.

As used herein, lymphoid cells include lymphoid cells that derived from any tissue in which lymphoid cells are present. In general lymphoid cells are removed from an individual who is to be treated. The lymphoid cells may be derived from a tumor, peripheral blood, or other tissues, such as the lymph nodes and spleen that contain or produce lymphoid cells.

As used herein, adoptive immunotherapy is a process whereby in vitro expanded lymphoid cells are transferred, administered or introduced into an individual or host.

When the lymphoid cells are cultured in vitro under appropriate conditions certain subpopulations thereof are selectively expanded. The expanded subpopulations of cells that are produced are herein referred to as in vitro expanded lymphoid cells. The subpopulation of cells is generally a heterogeneous mixture of cells having different phenotypes, but it may also consist of a homogeneous population of cells. The particular mixture of cells that are produced is a function of the starting material and the conditions under which such cells are generated. The expanded subpopulation can be used in adoptive immunotherapy protocols. Upon introduction into an individual who is being treated, the expanded subpopulation of cells specifically recognizes and in some manner mediate destruction of the host's afflicted cells and/or produces therapeutically effective agents. The conditions under which such cells are produced include growth in the presence of a cytokine or a mixture of cytokines, such as IL-2, IL-1, IL-6 and IL-4. If the lymphoid cells are cultured in the presence of a cytokine, then in vitro expanded subpopulations of lymphoid cells that are produced include activated lymphoid cells and, depending upon the source thereof and the cytokine used, may include LAK cells and TIL cells. If the lymphoid cells that are expanded in the presence of the cytokine are derived from a tumor, then the in vitro expanded lymphoid subpopulation of cells that is produced are referred to

as TIL cells. If the lymphoid cells are expanded in the presence of a cytokine and target antigen, then the cells that are produced are herein referred to as activated lymphoid cells. If the target antigen is disposed on a cancerous cell or is derived therefrom, then the in vitro expanded lymphoid subpopulation of activated lymphoid cells are TIL cells.

As used herein, therapeutically useful subpopulations of in vitro expanded lymphoid cells are those that can be used for adoptive immunotherapy.

As used herein, in vitro expanded lymphoid cells are cells that are cultured for use in methods of adoptive immunotherapy. Such cells constitute subpopulations of lymphoid cells that are produced when lymphoid cells are cultured in the presence of specific mediators that induce expansion of at least one particular subpopulation of lymphoid cells. Such subpopulations include those that are immunologically reactive with a diseased patient's affected cells. When such cells are used as therapeutic agents they are herein referred to as immunologically reactive lymphoid cells. TILs and LAK cells, when used for adoptive immunotherapy of cancer are examples of immunologically reactive cells. Other subpopulations of in vitro expanded lymphoid cells, include lymphoid cells that have been modified by genetic engineering to contain DNA that encodes proteins that are not normally produced by said lymphoid cells. Examples of such proteins include traceable foreign marker proteins and therapeutically effective substances, such as the CD4 protein to which HIV binds and anti-cancer agents. In addition, such cells may be genetically engineered to contain DNA

encoding drug resistance or drug sensitivity so that such cells may be selectively expanded or destroyed in vivo. Other subpopulations include lymphoid cells that have been cultured in vitro in the presence of specific target antigens. The target antigen or antigens may be disposed on the surface of a cell, such as a tumor cell, may be from cells or prepared synthetically and introduced into the tissue culture medium. Target antigens may be synthesized by the methods of peptide synthesis or by genetic engineering. In general in vitro expanded lymphoid cells are cells that have been cultured in the presence of a target antigen specifically recognize and in some manner mediate destruction of cells bearing the target antigen or deliver a therapeutic agent to cells that bear the target antigen.

As used herein, a target antigen is an antigen that is specifically recognized by a subpopulation of in vitro expanded lymphoid cells. An effective amount is at least one target antigen is an amount that is sufficient to select for the expansion of at least one subpopulation of in vitro expanded lymphocytes that specifically recognize said antigen.

As used herein, tumor-specific in vitro expanded lymphoid cells are cells that specifically recognize target antigens that are present on or in tumor cells. TIL cells are tumor specific lymphoid cells. As used herein a tumor-specific antigen is an antigen that is disposed on the surface or inside of a tumor cell. Tumor specific antigens may used in purified form, on irradiated tumor cells, or they may be obtained by purifying them from tumor cells or by synthesizing them in vitro by methods, such as genetic engineering.

As used herein, a growth promoting substance is a substance that in some manner participates in or induces cells to grow and/or divide. Growth promoting substances include mitogens and cytokines. Examples of growth promoting substances include the fibroblast growth factors, epidermal growth factor, the products of oncogenes, the interleukins, colony stimulating factors, and any other of such factors that are known to those of skill in the art.

As used herein, a mitogen is a substance that induces cells to divide and in particular, as used herein, are substances that stimulate a lymphocyte population in an antigen-independent manner to proliferate and differentiate into effector cells. Examples of such substances include lectins and lipopolysaccharides.

As used herein, a cytokine is a factor, such as lymphokine or monokine, that is produced by cells that affect other cells.

As used herein, a lymphokine is a substance that is produced and secreted by activated T lymphocytes and that affects other cell types. The tumor necrosis factor, the interleukins and the interferons are examples of lymphokines. A monokine is a substance that is secreted by monocytes or macrophages that affects other cells.

As used herein, a therapeutically effective number of <u>in vitro</u> expanded lymphoid cells is the number of such cells that is at least sufficient to achieve the desired therapeutic effect, when such cells are used in a particular method of adoptive immunotherapy. For example, a therapeutically effective amount of TILs for

a single treatment of a patient suffering from metastatic cancer is at least about 10^{10} to 10^{11} cells.

As used herein, a hollow cell fiber culture system consists of a hollow fiber bioreactor as well as pumping means for perfusing medium through said system, reservoir means for providing and collecting medium, and other components, including electronic controlling, recording or sensing devices. . A hollow fiber bioreactor is a cartridge that consists of a multitude of semi-permeable tube-shaped fibers encased in a hollow shell. The terms hollow fiber reactor and hollow fiber bioreactor are used interchangeably.

As used herein, the extra fiber space (EFS) is the space in which the cells grow within the shell of the hollow fiber bioreactor that is external to the semi-permeable fibers.

As used herein, the EFS cell supernatant is the medium in which the cells in the EFS are growing. It contains secreted cellular products, diffusible nutrients and growth promoting substances, such as lymphokines and cytokines, that mediate specific expansion of subpopulations of <u>in vitro</u> expanded lymphoid cells.

Thus, as used herein, a hollow fiber bioreactor consists of an outer shell casing, the semi-permeable fibers, and the EFS, which contains the cells and the EFS cell supernatant.

As used herein, EFS conditioned medium is the EFS cell supernatant after it has been centrifuged to remove any cells and particulate matter and dialyzed against tissue culture medium.

As used herein, selective medium is a tissue culture medium in which the <u>in vitro</u> expanded lymphoid

cells are generated that contains the desired growth promoting substance or substances and any other selective agents, such as target antigens or agents designed to select for growth of genetically engineered cells.

As used herein, complete AIM-V is a selective medium that consists of the proprietary formula AIM-V (GIBCO, Grand Island, N.Y.) and also contains 1000 units of IL-2 /ml. (provided by Cetus Corp., Emeryville, CA.), 10 μg. gentamicin/ml. (GIBCO), 50 μg. streptomycin/ml. (GIBCO), 50 μg penicillin/ml. (GIBCO), 1.25 μg. fungizone/ml. (Flow Laboratories, MacLean, VA.), 2.95 mg. glucose/ml. and 2 mM. glutamine (Flow Laboratories). Supplemented complete AIM-V consists of complete AIM-V that is supplemented with 20% AIM-V supernatant that is obtained from cultures of autologous LAK cells.

As used herein, AIM-V supernatant is prepared as described in Muul et al. (1986) J. Immunol. Methods 88: 265). Briefly, LAK AIM-V supernatant is prepared by growing peripheral blood lymphocytes in AIM-V or other suitable tissue culture medium in the presence of IL-2 for 2 to 3 days and removing the cells by centrifugation to obtain the supernatant.

Other suitable tissue culture media are well-known and readily available to those of skill in the art and may be readily substituted for AIM-V. For example, a medium that consists of a 50-50 mixture of complete AIM-V and RPMI having 10% heat-inactivated human serum, and further supplemented with LAK supernatant may be used.

As a first step when practicing any of the embodiments of the invention disclosed herein lymphoid cells that can be used to generate the desired

subpopulations of in vitro expanded lymphoid cells must be removed from an individual who is generally the patient who is to be treated using an adoptive immunotherapeutic method. Lymphoid cells are present in many tissues in the body, include PBL, lymph nodes, spleen cells, and any tissue in which an immune response is being mounted. The lymphoid cells that are contemplated for use in this invention are any whose development and growth may be directed by appropriate growth promoting substances to produce in vitro expanded lymphoid cells that are suitable for any method of adoptive immunotherapy.

The cells obtained from the patient are suspended in any cell culture medium that is suitable for sustaining the growth of such mammalian cells. Cell suspensions may be prepared from tumors or otherwise affected tissue or from lymphoid cells. Such media are readily available and the choice of an appropriate medium is well within the level of skill in the art. The cells are then plated into culture plates, or other suitable means known to those of skill in the art, for further expansion in an selective medium that also contains the growth promoting substance(s) that is(are) necessary for selective expansion of the desired subpopulation of immunoreactive lymphocytes. The medium may also contain target antigens. The cells are cultured and their numbers expanded until a sufficient number are obtained. Sometime at this point, the cells may be inoculated into a gas permeable bag and grown in selective medium containing the desired growth promoting substance and any other desired selective agents until at least about 10^8 cells are generated. Because the cells are cultured in

selective medium, the cells in suspension include primarily the subpopulation(s) of interest. The cells that have been generated are injected into a hollow fiber bioreactor, and cultured under conditions that are designed to expand the subpopulations thereof that can be used in methods of adoptive immunotherapy.

Hollow fiber bioreactors (abbreviated herein as HF) are known to those of skill in the art (see, e.g., Knazek et al. U.S. Patent Nos. 4,220,725, 4,206,015, 4,200,689, 3,883,393, and 3,821,087, which disclosures are herein incorporated by reference thereto). Hollow fiber bioreactors have been used for the growth of mammalian cells and for the production of biologically active products that are secreted thereby (see, e.g., Knazek et al., supra., see, also, Yoshida et al., U.S. Patent No. 4,391,912; Meyers et al., U.S. Patent No. 4,546,083; and Markus et al., U.S. Patent No. 4,301,249).

The hollow fiber bioreactor that is contemplated for use in the practicing of this invention contains a multitude of tube shaped semi-permeable membranes (hereinafter called fibers) that are encased in a hollow shell. Cultured cells grow and fill the spaces between the fibers and are fed diffuse of or the flow of nutrients from medium that is perfused through the lumina of said membranes. An example of a hollow fiber bioreactor that may be used in practicing this invention is the hollow fiber bioreactor, B3, Cellco Advanced Bioreactors, Inc., Kensington, MD, (see U.S. Application Serial No. 07/238,445, supra. for a complete description thereof). The bioreactor, B3, contains about 8000 tube-shaped, semi-permeable membranes, which provide a 1.1 m^2 surface area. The fibers, which are each approximately

250 μm in diameter, are pulled through a polycarbonate tube that is about 12 inches in length, and sealed at each end in such a manner that liquid only flows through the lumina of the fibers to exit at the opposite end of the shell. The fiber walls nominally restrict passage to substances having molecular weights less than a desired cut-off range. The fibers divide the cartridge into the extra-fiber space (EFS), typically about 50 ml. in volume, and the volume within the fiber lumina. The fibers and shell form a hollow fiber cartridge. Minimal bulk flow of liquid occurs within the extra-fiber space, which is also referred to as the extra-capillary or shell-side space.

If desired, prior to use, selected target antigens and/or the growth promoting substances may be bound to the fibers. The fibers must be selected so that the target antigen and/or growth promoting substance can bind thereto. Binding may be irreversible and may be accomplished by the use of cross-linking agents or other methods known to those of skill in the art or binding may be reversible, such as by absorption of the antigen or substance to the fiber.

The hollow fiber bioreactor is a component of a hollow fiber cell culture system. A typical hollow fiber cell culture system, such as the CELLMAX 100™ hollow fiber cell culture system (Cellco Advanced Bioreactors, Inc., Kensington, MD.), which is described in Knazek et al. U.S. Patent Application No. 07/238,445, supra., which disclosure is herein incorporated in its entirety by reference thereto, consists of a standard glass media bottle, which serves as the reservoir, stainless steel/Ryton gear pump, an autoclavable hollow

fiber bioreactor, which consists of the fibers and shell casing in which cells are cultured, and medical grade silicone rubber tubing, or other connecting means, which serves as a gas exchanger to maintain the appropriate pH and pO_2 of the culture medium. All components are secured to a stainless steel tray of sufficiently small dimensions to enable four such systems to fit within a standard tissue culture incubator. The pump speed and automatic reverse of flow direction are determined by an electronic control unit which is placed outside of the incubator and is connected to the pump motor via a flat ribbon cable which passes through the gasket of the incubator door. The pump motor is magnetically coupled to the pump and is lifted from the system prior to steam autoclaving. Tissue culture medium, which may also contain target antigens and/or growth promoting substances, such as IL-2, is drawn from the reservoir, pumped through the lumina of the hollow fibers, and then passed through the gas exchange tubing in which it is re-oxygenated and its pH readjusted prior to returning to the reservoir for subsequent recirculation. The flow rate can be increased as the number of cells increases with time. Typically the initial flow rate of the medium is adjusted to about 40 ml./min. and is increased over time to about 300 ml./min. The direction of perfusion of the medium through the hollow fiber lumina is periodically and automatically reversed, typically every ten minutes, in order to provide a more uniform distribution of nutrient supply, waste dilution, and cells within the space surrounding the hollow fibers.

The entire system is sterilized prior to cell inoculation and is designed for operation in a standard

air -CO_2 tissue culture incubator. Upon inoculation, the cells settle onto the surface of the hollow fibers, through which nutrients pass to feed the cells and into which metabolic waste products pass and are diluted into the large volume of the recirculating perfusate. The selected fiber should be semi-permeable to permit the passage of nutrients into the EFS and should be of a material on which or in the vicinity of which the cells are able to grow. The fibers are made of material, such as DEAE-cellulose or polypropylene, that is porous and suitable for the growth of mammalian cells. For example, cellulosic hollow fibers 12 inches in length, whose walls nominally restrict diffusion to substances having a molecular weight less than 3000 Daltons are suitable for use in practicing this invention. In some embodiments of this invention the tumor cells or target antigens are bound to the fibers, either reversibly or irreversibly, so that the lymphoid cells are constantly grown in the presence of the such antigens that are recognized by the immunoreactive cells. In other embodiments, the growth promoting substance is bound to the fibers. Binding may be reversible, such as by adsorption, or irreversible if a cross-linking agent is used to permanently affix the antigen or growth promoting substance to the fiber. Alternatively, the growth promoting substance and/or antigen may also be included in the perfusate and/or in the EFS.

A suspension of cells is inoculated into the extra-fiber space (EFS) of a hollow fiber bioreactor typically through one of two side ports. The lumina are perfused with cell culture medium, which contains diffusible nutrients and may also contain the growth

promoting substance(s), which specifically expand the subpopulation(s) of lymphocytes that can be used in adoptive immunotherapy and may also contain any target antigens.

Selection of the growth promoting substance or substances is a function of the subpopulation of lymphoid cells that is desired. Such selection is within the level of skill in the art and is dictated by the specific subpopulation of lymphoid cells that is desired. For example, if TIL cells are being grown, then IL-2, which functions in some manner in directing the growth, and possibility the development, of TIL cells from tumor tissues, must be included in the culture medium. Growth promoting substances, such as lymphokines, including IL-2, are available to those of skill in the art. Many, such as IL-2, have been cloned and expressed in biologically active form. Recombinantly produced growth promoting substances, such as recombinantly produced interleukins, are suitable for use in this invention. Means to clone DNA encoding such proteins and means to produce biologically active proteins from such cloned DNA are within the skill in the art. For example, interleukins 1 through 6 have been cloned. Various growth promoting substances and combinations thereof may be used to expand desired subpopulations of lymphoid cells.

In a typical embodiment of this invention the cells are cultured in the presence of at least one growth promoting substance that specifically expands at least one immunoreactive subpopulation of lymphoid cells and in any medium that is known to those of skill in the art to be suitable for the growth of mammalian cells <u>in vitro</u>.

It is well-within the level of skill in the art to select an appropriate culture medium. The growth promoting substance that is contemplated for use in this invention is selected for its ability to expand in vitro subpopulations of lymphoid cells that specifically recognize and mediate destruction of a patient's afflicted cells, such as cancerous or virally infected cells. In other embodiments of this invention the cells are grown in the presence of at least one target antigen in addition to at least one growth promoting substance. In another embodiment of this invention the lymphoid cells are isolated, modified by genetic engineering methods, injected into the extra-fiber space of a hollow fiber bioreactor and cultured under conditions that are designed to expand the genetically modified subpopulation thereof.

After inoculation, the culture medium is continuously perfused through the hollow fiber bioreactor by means of externally applied pressure, such as a pump. A glass reservoir, the hollow fiber bioreactor, and pumping means are connected by tubing, typically silicone rubber, which simultaneously serves as a membrane gas exchanger to replenish oxygen and, if the medium is buffered with bicarbonate, to maintain the pH via CO_2 transport into the perfusion medium. Medium that is buffered with systems other than bicarbonate do not necessarily require CO_2 in the incubator.

The in vitro expanded lymphoid cells grow and divide and pile up upon each other until they fill up a substantial portion of the extra-fiber space to form nearly solid masses of cells. As the cells grow and divide, the perfusate can be replaced and the EFS can be

periodically drained.

The perfusing medium can be replaced by replacing the reservoir bottle. After growth of the cells has been established, it has been discovered that it is not necessary to include human serum albumin in the perfusion medium. The EFS can be drained periodically to harvest the supernatant and/or to sample the cells. When the EFS is drained, any cells that have been drained can be recovered and re-inoculated into the hollow fiber bioreactor suspended in complete AIM-V or other serum protein-containing medium.

The in vitro expanded lymphoid cells are cultured in the hollow fiber bioreactor until the EFS contains at least about 10^{10} to 10^{11} cell. The cells can be harvested by shaking the bioreactor and pouring the cells along with the EFS medium into a side port bottle. In addition, the EFS cell supernatant, which is rich in non-or poorly-diffusible cellular products, including useful biologically active agents, such as IL-2 receptors and other growth promoting substances that are useful for expanding desired subpopulations of lymphoid cells in vitro, can be recovered for further processing in order to purify or partially purify said biologically active agents. The cells can be spun down using a centrifuge or by any other means known to those of skill in the art to yield a cell pellet and the EFS cell supernatant, which is enriched in biologically active molecules, such as IL-2 receptors and growth promoting substances.

After harvesting the cells, the growth of cells remaining in the bioreactor can be re-instituted by resuming perfusion of the culture medium, which contains the growth promoting substance(s). The cells will then

continue to divide and can be harvested. This step can be repeated a plurality of times.

After harvest and pelleting of the cells, the EFS cell supernatant can be dialyzed against fresh tissue culture medium in order to produce EFS conditioned medium, which can then be further processed or used directly or diluted to stimulate the growth of cells, such as TIL cells.

In one typical procedure using the methods of this invention a tumor is excised from a patient suffering from malignant melanoma, minced into small pieces and suspended in RPMI 1640 tissue culture medium (Biofluids, Rockville, MD.) that contains 10 mg. collagenase/ml., 1 mg. deoxyribonuclease/ml., and 2.5 units of hyaluronidase/ml. (Sigma). All operations in which the cells are manipulated are performed using sterile techniques in a laminar flow hood. The suspension is stirred overnight, filtered through Nitex Mesh and resuspended in LAK supernatant supplemented complete AIM-V medium, which contains IL-2, cultured, and is then plated onto culture plates at a density of about 5×10^5 cells/ml. After about one week or when the cell densities of TIL cells reached about $1-2 \times 10^6$ cells/ml., the cells are replated in fresh medium at densities of about 5×10^5 cells/ml and after further growth the cells, which are adjusted to a density of about 2×10^6 cells/ml., approximately 50 ml. are inoculated into a hollow fiber bioreactor. Prior to use the hollow fiber culture system is steam autoclaved, continuously perfused with 1.3 liters of recirculating deionized water, drained, flushed, and perfused with complete AIM-V medium in both the EFS and perfusate pathways.

The inoculated bioreactor is transferred to a standard incubator where it is perfused with complete AIM-V. Incubation continues for at least about 10 days up to about 30 days until the number of cells in the bioreactor reaches a therapeutically effective amount of cells, about 10^{10} to 10^{11} cells. During the incubation period the reservoir containing the perfusing medium is changed in order to maintain a sufficiently high concentration of glucose and other diffusible nutrients in the EFS. If desired, the EFS can be periodically drained during the incubation period in order to sample the cells or to collect the EFS cell supernatant.

When the desired cell density is reached the cells are harvested by vigorously shaking the hollow fiber bioreactor and draining the EFS. The cells are pelleted and the EFS cell supernatant collected for further processing. The harvested cells possess the morphological and biological characteristics of TIL cells.

The EFS cell supernatant can be dialyzed against fresh tissue culture medium to produce EFS conditioned medium and added to newly seeded cells or order to stimulate the growth of the newly seeded cells. The EFS can also be added to the hollow fiber bioreactor, after harvesting the cells, when growth is re-instituted in order to stimulate the growth of the cells remaining therein.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLE 1

Prior to use eight hollow fiber culture systems were steam autoclaved at 121° C for 20 minutes and then perfused with 1.3 liters of deionized water overnight at 37° C. The perfusion pathway and extra-fiber space of each system were drained and flushed with complete AIM-V medium before replacing the reservoir bottle with a fresh warmed 1 liter bottle of complete medium. All operations were performed in a sterile laminar flow hood.

Tumors were excised from 8 patients (listed in column 1 of Table 1) who had metastatic melanoma. The tumors were sterilely transported from the surgical suite to a laminar flow hood, where the cells from each patient were processed separately. The tumorous tissue from each patient was minced into 1 mm. to 2 mm. pieces and suspended in approximately 200 to 500 ml. of RPMI 1640 tissue culture medium (Biofluids, Rockville, MD.) that contained 10 mg. collagenase/ml., 1 mg. deoxyribonuclease/ml., and 2.5 units of hyaluronidase/ml. (Sigma). Each suspension was gently stirred overnight at room temperature and then filtered through sterile Nitex mesh, washed twice, and resuspended in either LAK supernatant supplemented complete AIM-V medium or in a medium that consisted of a 50-50 mixture of complete AIM-V and RPMI having 10% heat-inactivated human serum, and 20% LAK supernatant and IL-2 (1000 units/ml.).

Each suspension of cells was plated into 6 well culture plates (Costar Corp., Cambridge, MA.) at densities of about 5×10^5 cells/ml. After about one week or when the cell densities of TIL cells reached about $1-2 \times 10^6$ cells/ml., the cells were replated in fresh medium at densities of 5×10^5 TIL/ml. After further growth the

cells were diluted into 0.5 to 1.5 liters of complete AIM-V medium to a density of 5 x 10^5 cells/ml. The entire volume was then injected into a 1.5 liter polyolefin culture bag (PL-732 plastic, Fenwal Laboratories, Deerfield, IL.) and incubated in a flat position on a perforated shelf without agitation at 37° C for 3-4 days in a humidified 5% CO_2/air incubator. As the cells multiplied, they were periodically diluted 1:3-4 in complete AIM-V medium in new bags. Upon inoculation into the bags the cells, after an initial lag phase, entered a period of exponential growth.

During the exponential growth phase of the cells in the bags, a small volume of the cell suspension was withdrawn 17 to 33 days after excision of the tumor, centrifuged at 400 x g. for 10 minutes at room temperature. The pelleted cells were resuspended in complete AIM-V medium in the 100 ml. glass side port bottles The cells from each of the 8 patients with metastatic melanoma were then inoculated into the hollow fiber culture system. The cells that continued to be cultured in the bags served as controls to which the cells cultured according to the methods of this invention were compared.

Cells that had been derived from each patient (B, M, S, K, J, H, W, and G) were each inoculated into a hollow fiber cartridge and into a bag. Inocula ranged from 0.35 to 4.3 x 10^8 cells. The cells were injected into the extra-fiber space of the hollow fiber bioreactor through the side-ports that are each connected to the 100 ml glass bottle in which the TIL cells were suspended. The bottles were gently pressurized with a 20 ml. plastic syringe to force the cell suspension into the extra-fiber

space. The cells settled on or near the fibers from which they received nutrient support by diffusion flow from the perfusate. Simultaneously, low molecular weight metabolites diffused away from the cells and through the fiber where they were diluted by the perfusate.

Two of the 8 cultures, S and J (see Table I) had stopped growing in both the hollow fiber cultures and in the control bags. Because the hollow fiber cultures and bag cultures were maintained in separate incubators, it would appear that the failure to grow was related to the cells and not to the method by which the cells were being cultured. The remaining 6 TIL cultures grew well in both the hollow fiber culture system and the bags. Fig. 1 depicts a growth curve for the cells that were derived from patient W.

The system was operated in a 37° C, humidified, 5%, CO_2/air incubator. During the course of the process, the reservoir bottle, which contained the perfusion medium, complete AIM-V, was periodically replaced when its glucose concentration decreased to the range of 100-150 mg./dl. Other nutrients, as well as glucose, are replenished by replacing the medium containing bottle with a pre-warmed bottle containing fresh medium. The number of times that the bottles were replaced is indicated in column 8 of Table I. Thus, the amount of medium used for each culture depended upon the amount of glucose consumed thereby and ranged from 8 to 27 liters per hollow fiber culture system, which is equivalent to about 2.4 to 6.6 liters of complete AIM-V per 10^{10} TIL harvested (See Table I, infra.). The time required to replace a reservoir bottle was less than 5 minutes.

It was discovered that once the cell culture was

initiated in the hollow fiber bioreactor, it was no longer necessary to add human serum albumin to the perfusion medium. The cells grew substantially as well in the absence of human serum albumin in the perfusate as in the presence thereof.

The extra fiber space was drained periodically either to harvest the culture medium, which is enriched in non-diffusible products that are secreted by TIL cells, or to sample the cells in order to evaluate them for number and functional characteristics. The number of times the EFS was drained for each culture is indicated in column 9 of Table I. Medium in the EFS was replaced on the average, except for culture G, every 2.2 days. The EFS of culture G was replaced 2 and 0 times during the respective 14 and 21 day culture periods.

The extra fiber space was drained in a laminar flow hood by gently draining the extra-fiber fluid into one of the two loading side port bottles. The medium that was drained from the EFS was centrifuged at room temperature for 10 minutes at 200 x g, the cell pellet was resuspended in fresh complete AIM-V medium and re-inoculated into the EFS of the hollow fiber bioreactor so that any TIL that had been flushed out would not be discarded. The EFS cell supernatant was saved for further processing. Draining, pelleting, resuspending, and re-inoculation of the bioreactor took 20-30 minutes.

As the number cells in the bioreactor increased over time the flow rate of the perfusate was increased from 40 ml to 300 ml./minute. The direction of perfusion was reversed every ten minutes. Incubation was continued for 14 to 32 days (see Table I) at which time the cells were ready to be harvested.

EXAMPLE 2

After 14 to 32 days the entire hollow fiber culture system was removed from the incubator an placed in a laminar flow hood. The electronic control unit was then reconnected to the pump motor and perfusion continued at a rate of about 40 ml./minute in order to prevent the cultured cells from becoming anoxic during the harvest procedure. Approximately 1/3 of the extra-fiber medium was drained by gravity into a loading side port bottle. The hollow fiber reactor was then shaken vigorously in order to detach any cells from the fibers. The remaining medium and cells in suspension were drained into the side-port bottle. The procedure was then repeated three times by adding 35 ml. of fresh complete medium into the side-port and injecting it into the extra-fiber space prior to shaking the bioreactor. The last two washes were accomplished by shaking the cartridge with a hand held vibrator (Oster Corp., Milwaukee, WI.) for 1 minute to remove the more firmly attached cells. The cells from the hollow fiber cartridge were contained in a final volume of about 155 to 250 ml.; whereas, the equivalent number of cells grown the bags are contained in at least about 10-20 gas permeable bags or about 15-30 liters of medium.

After harvesting, the cells from each bioreactor were centrifuged to form a final pellet, which is ready for subsequent use. The entire harvesting procedure took about 30 minutes and about 95% of the cells in the bioreactor were recovered. Each hollow fiber bioreactor yielded from 1.5-5.4 x 10^{10} cells.

The data for the cells from each of the 8 patients is summarized in Table I. The number of cells

harvested from each hollow fiber culture system is indicated in column 5.

EXAMPLE 3

1.5×10^{10} TIL from patient G were harvested from the bioreactor on day 30, 15 days after the bioreactor was inoculated. Perfusion of the bioreactor with complete AIM-V was re-instituted and the residual cells within the EFS continued to divide and grow and yielded an additional 1.5×10^{10} cells on day 21 (see Table I and Fig. 2). Perfusion was again re-instituted and the residual cells were once again permitted to expand for a third harvest of 2.1×10^{10} cells on day 73.

After the final harvest, the entire CELLMAX™ hollow fiber culture system was steam-autoclaved at 121° C for 20 minutes. The system was then flushed with deionized water and the silicone rubber tubing and hollow fiber bioreactor were discarded. Cleaning, reassembly, and re-autoclaving with fresh tubing and bioreactor in place was performed before the system was reused for a different patient.

EXAMPLE 4

The in vitro functional characteristics of cell suspensions harvested or sampled from the bioreactors were compared to the corresponding suspensions harvested or sampled from the bags.

Cells were assayed periodically and after harvesting for viability by diluting a sample in trypan blue/normal saline solution (SIGMA) to a final concentration of 0.04% gm./dl. Viability was estimated by dye exclusion as observed under microscopic examination. The viability of the cells, which is presented in Table I varied from patient to patient. The

average viability of the cells grown in the hollow fiber culture system was greater than 90%.

During the course of culturing, aliquots of perfusate were assayed to determine the glucose and lactate concentrations (Yellow Springs Instrument, CO., Yellow Springs, OH.), which permitted their rates of consumption and production, respectively, to be measured. See Figs. 1 and 2. The rates of glucose consumption by TIL from different patients varied between 0.45 to 2.2 gms. per 10^{10} TIL/24 hours (Table I, col. 7). The rates of lactic acid production approximated the rates of glucose consumption. Such rates exhibited logarithmic increases over time, doubling every 1.5-3.6 days. These double times were commensurate with the doubling times, 1.5 to 3.2 days, of the cells grown in the bags.

Cytotoxicity of the cells was assessed by a chromium release assay tested against targets that consisted of autologous tumor cells, allogeneic tumor cells, the NK-sensitive, K562 erythroleukemia cell line, and the NK-resistant Daudi B-cell lymphoma line (see, e.g., Topalian et al. (1987) J. Immunol. Meth. 102: 127). The results of the cytoxicity studies set forth in Table II, which presents the results of measurements of the cytolytic capacities of three dilutions of TIL taken from simultaneous aliquots of bag and hollow fiber cultures.

The percentage of specific lysis of K562 cells, Daudi cells, autologous tumor cells, and allogeneic tumor cells by various samples of the TIL cells from patients M,K, H, W, and G taken at different times during the growth period was measured by a chromium release assay. The percentage specific lysis by LAK cells, which possess

lytic capacity for all cell lines as well as autologous and allogeneic tumor cells was also measured.

Because the K562 cell line is NK-sensitive it serves to assess the activity of NK cells in the in vitro expanded lymphoid cells that were derived from each patient. $NKHI^{+}$ cells, are responsible for non-specific killer activity. The Daudi cell line, which is NK-resistant but LAK sensitive, indicates the relative amount of LAK activity present in a given sample.

Approximately 10^8 target cells were labelled with 400 μCi of sodium $^{51}CrO_4$ (New England Nuclear, Boston, MA.) in a 0.7 ml. volume for 1 hour, washed three times, incubated for an additional 30 minutes at 37° C and washed twice before use. Serial dilutions of the sampled effector cells were plated with 5 x 10^8 target cells in triplicate at ratios of effector:target cell of 80:1, 20:1, and 5:1 in a total of 150 μl of culture medium in 96 well round bottom microtiter plates (Costar Corp.) and incubated for 4 hours. Supernatants were then harvested with the Skatron-Titertek system (Skatron, Lier, Norway) and counted in a gamma counter (LKB Instruments, Gaithersburg, MD.). The amount of radioactivity that is released by spontaneous target lysis was determined by incubation of tumor target cells in the absence of effector cells. The maximal amount of radioactivity released, which represents maximal cell lysis, was measured by incubating the target with 2% SDS.

Percentage of specific lysis, thus, equals:

$$\frac{\text{(experimental release - spontaneous release) x 100}}{\text{maximal release -spontaneous release}}$$

Although the experiment was somewhat limited in scope, it appears from the data presented in Table II

that the relative activities of the different cell types in a TIL cell preparation that were expanded in the hollow fiber bioreactor varied from patient to patient and also over time in a single patient. See e.g., the data obtained for the W-TILs set forth in Table II in which the first set of measurements were obtained at day 7 after inoculation into the hollow fiber bioreactor and the second set were obtained at day 28. If, however, the data for each sample taken from either the bags or HF for a single patient's cells taken on the same day are compared, certain consistent differences between the cells cultured in the HF and bags become apparent. NK activity and LAK cell activity appear to be lower for the cell populations grown in the HF than for the cells grown in the bags. Further, these differences do not seem to correlate with a concomitant and equivalent decrease in TIL activity. Thus, the cells grown by the methods of this invention appear to contain a lower percentage non-TIL-associated activity.

The phenotypes of the sampled and harvested cells from the bags and hollow fiber reactors were also compared. The results of these experiments for patients K and G are summarized in Table III.

Sampled or harvested cells were washed with cold staining necium (Hank's buffered saline solution without phenol red that contains 5% heat-inactivated fetal calf serum and 0.02% sodium azide) and resuspended in medium at concentrations of about 1×10^6 to 1×10^7 cells/ml. Undiluted fluorescein-conjugated monoclonal antibodies against human mononuclear cell antigens (Becton-Dickinson, Mountain View, CA.) were added to 100 μl volumes of cell suspension at a concentration of 5%

(v/v). Monoclonal antibodies were used to ascertain what surface antigens are present on the cell surface and to, thus, functionally characterize the cells. The antibodies tested were anti-Leu-4, which reacts against T cells, anti-Leu-3a, which reacts against T helper/inducer cells, anti-Leu-2b, which reacts against T cytotoxic/suppressor cells, anti-Leu-5b, which reacts against E rosette receptor-bearing cells, anti-Leu-7, which reacts against NK and some T cells, anti-Leu-11a, which reacts against NK cells and neutrophils, anti-Leu-14 and anti-Leu-16, which react against B cells, anti-HLA-DR, which reacts against activated T cells, B cells, and monocytes/macrophages, anti-IL-2 receptor, which reacts against activated T cells, and anti-Leu-M3, which reacts against monocytes/macrophages. As negative controls the antibodies used included anti-Thy-1.2, which reacts against murine T cells, and phycoerthrosin. After staining for 30-60 minutes at 4° C, the cells were washed with staining medium, fixed with 1% paraformaldehyde , washed again, and resuspended in staining medium. Cells that were so-labeled were stored at 4° C for 1-7 days. Fluorescence analysis was performed using a FACS 440 microfluorometer that had been interfaced with a Consort 40 computer (Becton-Dickinson).

The results of these experiments indicate that the surface antigen profile of the cells grown by the method of this invention and those grown in the bags are substantially identical. Surface antigens present on the surface of cells grown in bags were also present on those grown by the methods of this invention and the surface antigens that were substantially absent on the cells grown in the bags were absent from the cells grown

in the bags were also substantially absent from the surface of cells that were grown by the methods of this invention.

TABLE I

TIL	HF* inoc days	days in cult.	Inoculum $x10^{-8}$	Harvest $x\ 10^{-10}$	Viability %	Glucose gm/d.	#resvr. chnge	#EFS chnge	Tot.l med.	Med./10^{10} harv.
B	33	21	0.5	4.5	85	3.6	12	10	11.3	2.5
M	31	32	0.35	4.1	97	3.5	25	14	27	6.6
S			1.3	---						
K	58	29	10.0	26ml.		1.9	12	10	13	
J			0.4	---						
H	24	26	1.8	2.8	91	1.7	9	12	10	3.6
W	24	22	4.3	5.4	93	2.4	12	7	13	2.4
G	17	14	1.0	1.5	87	2.9	7	2	8	5.0
G	31	21	Residual	1.5	89	3.3	8	0	8.8	5.8

* HF = hollow fiber culture system.
TIL = patient from which tumor was resected for production of TILs; the cells from patients S and J failed to grow in either the bags or HF.
HF inoc. days = number of days after the tumor was excised from patient that cells inoculated into HF device.
Days in cult. = days in culture in HF before harvest.
Inoculum x 10^{-8} = number of cells inoculated.
Viability = percentage viable at harvest.
Glucose gm/d. = glucose consumption rate by cells at harvest.
resvr. chnge = number of times medium in reservoir was changed during course of HF culture.
EFS chnge= number of time the extra fiber space changed during course of HF culture.
Tot. l med. = total liters of culture medium used by HF culture.
Med./10^{10} harv. = liters of culture medium used per 10^{10} cells harvested.

TABLE II

Effector: Target cell Ratio	Target cell K562 80:1	20:1	5:1	Target cell Daudi 80:1	20:1	5:1	Tumor cell lysis: Autologous 80:1	20:1	5:1	Tumor cell lysis: Allogeneic 80:1	20:1	5:1
Effector cell:												
LAK	44.4	12.9	6.9	28.4	9.1	5.7						
M-TIL (HF)	6.6	5.3	.8	2.5	1.1	4.1						
K-TIL (bag)	58.7	33.0	12.5	19.1	11.5	3.7						
K-TIL (HF)	21.6	11.2	7.4	4.8	5.0	4.5						
LAK	65.2	23.0	7.3	43.6	14.1	8.4	17.2	8.9	4.3	4.6	1.1	3.6
H-TIL (Bag)	7.4	5.8	.8	2.6	5.6	6.6	50.2	57.1	33.7	6.6	.1	-7.3
H-TIL (HF)	2.3	.9	.7	-.8	2.8	2.8	49.9	42.9	39.6	.8	5.0	- .6
K-TIL (HF)	4.2	1.3	.8	1.8	-2.1	-3.1				3.6	4.9	.1
LAK	81.0	44.0	18.0	38.8	18.6	6.0	5.1	.9	2.0	9.1	6.6	.9
W-TIL (Bag)	30.5	24.5	22.7	64.0	53.2	29.2	30.6	14.9	3.2	4.8	6.8	4.5
W-TIL (HF)	22.5	11.2	12.5	55.1	45.0	19.8	19.4	11.9	4.4	2.0	4.1	5.8
LAK	86.8	75.6	27.4	69.8	45.8	17.2	34.3	13.6	4.1	26.0	11.7	4.5
W-TIL (Bag)	15.0	6.9	.9	72.2	53.1	21.7	15.4	7.9	2.8	5.5	12.7	2.5
W-TIL (HF)	8.6	2.5	.3	55.9	41.3	17.0	11.5	0	2.0	3.9	5.4	5.0
LAK	74.3	56.1	49.9	59.7	45.9	40.0	69.9	41.3	18.1	37.1	43.1	17.7
G-TIL (Bag)	13.5	8.5	5.2	21.3	4.9	2.6	51.1	32.0	22.3	3.9	2.9	2.5
G-TIL (HF)	11.1	10.1	6.1	3.9	9.6	7.6	50.4	40.4	38.2	3.4	3.2	3.6

TABLE III

Phenotypic Profile

	K-HF	K-Bag	G-HF	G-Bag
Leu 2	95.9	90.4	73.8	83.1
3	1.1	2.0	13.1	14.3
4	94.1	94.7	85.3	91.4
7	4.2	11.7	2.2	4.6
11	.6	.8	2.2	1.4
15	.3	9.5	3.7	0.4
16	3.7	4.0	0.1	0.8
19	8.3	20.4	6.5	7.1
m3	- .4	.3	.6	1.1
HLA-DR	80.7	85.6	89.3	91.1
TAC	2.4	4.7	27.3	30.4

EXAMPLE 5

As in Example 1, a hollow fiber culture system is steam autoclaved at 121° C for 20 minutes and then perfused with 1.3 liters of deionized water overnight at 37° C. The perfusion pathway and extra-fiber space of each system are drained and flushed with complete AIM-V medium before replacing the reservoir bottle with a fresh warmed 1 liter bottle of complete medium. All operations are performed in a sterile laminar flow hood.

A tumor is excised from a cancer patient and is sterilely transported from the surgical suite to a laminar flow hood, where the cells from the tumor are minced into 1 mm. to 2 mm. pieces and suspended in approximately 200 to 500 ml. of RPMI 1640 tissue culture medium (Biofluids, Rockville, MD.) that contains 10 mg. collagenase/ml., 1 mg. deoxyribonuclease/ml., and 2.5 units of hyaluronidase/ml. (Sigma). The suspension is gently stirred overnight at room temperature and then filtered through sterile Nitex mesh, washed twice, and resuspended in LAK supernatant supplemented complete AIM-V medium as described in Example 1.

A portion of the suspension, at least about 50 ml., is irradiated with X-rays or other suitable means for a time sufficient to inactivate the tumor cells. The irradiated suspension in a cryoprotective media, well known to those knowledgable in the field, is stored in liquid nitrogen. Shortly before use, the suspension is warmed to about 37° C.

The remaining portion of suspended cells is plated into a 6 well culture plates (Costar Corp., Cambridge, MA.) at densities of about 5×10^5 cells/ml and after about one week or when the cell densities of TIL

cells reach about 1-2 x 10^6 cells/ml., the cells are replated in fresh medium at densities of about 5 x 10^5 cell/ml and cultured until at least about 10^7 to 10^8 cells are produced. The cells are pelleted and resuspended in the suspension that contains the irradiated tumor cells and the volume is adjusted to about 100 ml. with complete AIM V medium. The mixture is inoculated as described in Example 1 into the EFS of the hollow fiber culture system. The cells settle on or near the fibers.

The culture system is operated as described in Example 1. Initially the perfusion medium is complete AIM-V, which is periodically replaced when its glucose concentration decreased to about 1 to 1.5 grams/liter. Once the cell culture is established in the hollow fiber bioreactor, it is no longer necessary to add human serum albumin to the perfusion medium.

As the number cells in the bioreactor increased over time the flow rate of the perfusate is increased from 40 ml to 300 ml./minute. The direction of perfusion is reversed every ten minutes. Incubation is continued for 14 to 32 days at which time the cells are harvested as described in Example 1.

EXAMPLE 6

As in Example 1, a hollow fiber culture system is steam autoclaved at 121° C for 20 minutes and then perfused with 1.3 liters of deionized water overnight at 37° C. The perfusion pathway and extra-fiber space of each system are drained and flushed with complete AIM-V medium before replacing the reservoir bottle with a fresh warmed 1 liter bottle of complete medium. All operations are performed in a sterile laminar flow hood.

A tumor is from a cancer patient and is sterilely transported from the surgical suite to a laminar flow hood, where the cells from the tumor are minced into 1 mm. to 2 mm. pieces and suspended in approximately 200 to 500 ml. of RPMI 1640 tissue culture medium (Biofluids, Rockville, MD.) that contains 10 mg. collagenase/ml., 1 mg. deoxyribonuclease/ml., and 2.5 units of hyaluronidase/ml. (Sigma). The suspension is gently stirred overnight at room temperature and then filtered through sterile Nitex mesh, washed twice, and resuspended in LAK supernatant supplemented complete AIM-V medium as described in Example 1.

A portion of the suspension, at least about 50 ml., is removed. The suspension mixed with microcarrier beads to which said cells bind to form a slurry. The slurry is irradiated with X-rays for a time sufficient to inactivate the tumor cells. The slurry is then introduced into the bioreactor and the microcarrier beads are permitted to settle onto or near the fibers.

Alternatively, the portion of the suspension of tumor cells is irradiated with X-rays for a time sufficient to inactivate the tumor cells' growth. The suspension of tumor cells are then introduced into the EFS of the HF bioreactor and are permitted to settle onto or near the fibers. A non-toxic cross-linking agent introduced into the EFS and the tumor cells are irreversibly bound to the fibers. After cross-linking the EFS is drained and perfused with complete AIM-V medium.

The remaining portion of suspended cells is plated into a 6 well culture plates (Costar Corp., Cambridge, MA.) at densities of about 5×10^5 cells/ml and after about one week or when the cell densities of TIL

cells reach about 1-2 x 10^6 cells/ml., the cells are replated in fresh medium at densities of about 5 x 10^5 cell/ml and cultured until at least about 10^7 to 10^8 cells are produced. The cells are pelleted and resuspended in the suspension that contains the irradiated tumor cells and the volume is adjusted to about 100 ml. with complete AIM V medium. The mixture is inoculated as described in Example 1 into the EFS of the hollow fiber culture system. The cells settle on or near the fibers.

The culture system is operated as described in Example 1. Initially the perfusion medium is complete AIM-V, which is periodically replaced when its glucose concentration decreased to about 1 to 1.5 grams/liter. Once the cell culture is established in the hollow fiber bioreactor, it is no longer necessary to add human serum albumin to the perfusion medium.

As the number cells in the bioreactor increased over time the flow rate of the perfusate is increased from 40 ml to 300 ml./minute. The direction of perfusion is reversed every ten minutes. Incubation is continued for 14 to 32 days at which time the cells are harvested as described in Example 1.

EXAMPLE 7

As in Example 1, a hollow fiber culture system is steam autoclaved at 121° C for 20 minutes and then perfused with 1.3 liters of deionized water overnight at 37° C. The perfusion pathway and extra-fiber space of each system are drained and flushed with complete AIM-V medium before replacing the reservoir bottle with a fresh warmed 1 liter bottle of complete medium. All operations are performed in a sterile laminar flow hood.

A tumor is from a cancer patient and is sterilely transported from the surgical suite to a laminar flow hood, where the cells from the tumor are minced into 1 mm. to 2 mm. pieces and suspended in approximately 200 to 500 ml. of RPMI 1640 tissue culture medium (Biofluids, Rockville, MD.) that contains 10 mg. collagenase/ml., 1 mg. deoxyribonuclease/ml., and 2.5 units of hyaluronidase/ml. (Sigma). The suspension is gently stirred overnight at room temperature and then filtered through sterile Nitex mesh, washed twice, and resuspended in LAK supernatant supplemented complete AIM-V medium to as described in Example 1.

The suspension of cells is plated into a 6 well culture plates (Costar Corp., Cambridge, MA.) at densities of about 5 x 10^5 cells/ml and after about one week or when the cell densities of cells reach about 1-2 x 10^6 cells/ml., the cells are replated in fresh medium at densities of about 5 x 10^5 cell/ml and cultured until at least about 10^7 to 10^8 cells are produced. The cells are pelleted and resuspended in complete AIM V medium that additionally contains about 1000 units/ml. of IL-4. The is inoculated as described in Example 1 into the EFS of the hollow fiber culture system. The cells settle on or near the fibers.

The culture system is operated as described in Example 1. Initially the perfusion medium is either complete AIM-V or complete AIM-V that also contains 1000 units/ml. of IL-4. The perfusion medium is periodically replaced when its glucose concentration decreased to about 1 to 1.5 grams/liter. Once the cell culture is established in the hollow fiber bioreactor, it is no longer necessary to add human serum albumin to the

perfusion medium.

As the number cells in the bioreactor increased over time the flow rate of the perfusate is increased from 40 ml to 300 ml./minute. The direction of perfusion is reversed every ten minutes. Incubation is continued for 14 to 32 days at which time the cells are harvested as described in Example 1.

EXAMPLE 8

After removing the cells, which have been cultured as in Example 1, and the EFS medium from the hollow fiber bioreactor, the EFS and cells were centrifuged at 200 x g to pellet the cells. The EFS cell supernatant was dialyzed against fresh complete AIM V medium for 24 hours at 4° C to produce what is herein called EFS conditioned medium. 10^4 of the harvested cells were seeded in flasks and grown in duplicate in either 5 ml. of EFS conditioned medium at dilutions of EFS conditioned medium with complete AIM V of 1:10 and 1:100 EFS or in 5 ml. of dialyzed complete AIM V. It was found that the EFS conditioned medium stimulated growth of the cells. The data is presented in Table IV.

TABLE IV

STIMULATION OF GROWTH OF TIL BY EFS CELL SUPERNATANT

	DILUTIONS OF EFS	
	1:100	1:10
Growth in EFS conditioned medium	20	12.7
NO. CELLS X 10^{-4}	16.8	12.7
Growth in dialyzed Medium	9.2	10.0
NO. CELLS x 10^{-4}	9.0	13.2

Since modifications will be apparent to those of skill in the art, it is intended that this invention be limited only by the scope of the appended claims.

We claim:

1. A method for the production of in vitro expanded lymphoid cells that can be used in adoptive immunotherapy, comprising:

(a) inoculating the extra fiber space of a hollow fiber bioreactor that is a component of a hollow fiber culture system with a suspension of lymphoid cells;

(b) perfusing said bioreactor with tissue culture medium that contains an effective amount of at least one growth promoting substance that specifically expands a therapeutically useful subpopulation of said lymphoid cells, wherein said effective amount is an amount sufficient to effect said specific expansion, said tissue culture medium sustains the cell division and growth of said subpopulation, and said therapeutic use is adoptive immunotherapy; and

(c) culturing said cells in said bioreactor in the presence of said growth promoting substance for a time sufficient to obtain a therapeutically effective number of said in vitro expanded lymphoid cells; wherein said therapeutically effective number is the number of said cells that is at least sufficient to achieve a therapeutic effect when said cells are used in adoptive immunotherapy.

2. The method of claim 1, wherein the growth promoting substance is at least one substance selected from the group consisting of mitogens and cytokines.

3. The method of claim 2 wherein said cytokine is at least one cytokine selected from the group of cytokines consisting of interleukin-1, interleukin-2, interleukin 4, and interleukin 6.

4. The method of claim 1, further comprising harvesting the contents of the extra fiber space of the bioreactor, wherein said contents include said in vitro expanded lymphoid cells and the extra fiber space cell supernatant.

5. The method of claim 3, further comprising harvesting the contents of the extra fiber space of the bioreactor, wherein said contents include said in vitro expanded lymphoid cells and the extra fiber space cell supernatant.

6. The method of claim 4, further comprising re-instituting perfusion of said bioreactor and culturing the residual cells that remain in the extra fiber space of said bioreactor after said first harvest for a time sufficient to obtain a therapeutically effective amount of said cells; wherein said perfusion and harvesting are re-instituted at least once.

7. The method of claim 5, further comprising re-instituting perfusion of said device and culturing the residual cells that remain in the extra fiber space of said bioreactor after said first harvest for a time sufficient to obtain a therapeutically effective amount of said cells; wherein said perfusion and harvesting are re-instituted at least once.

8. The method of claim 1, wherein said medium also contains an effective amount of at least one target antigen, wherein said effective amount is at least sufficient to select for the expansion of at least one subpopulation of in vitro expanded lymphocytes that specifically recognize said antigen.

9. The method of claim 8, wherein said at least one of said target antigens is selected from the group

consisting of tumor specific antigens.

10. The method of claim 8, wherein target antigen is only in the medium in the extra fiber space of said bioreactor.

11. The method of claim 10, wherein said at least one of said target antigens is selected from the group of tumor specific antigens.

12. The method of claim 4, further comprising pelleting and removing the cells from said contents of the extra fiber space to produce an extra fiber space cell supernatant.

13. The method of claim 12, further comprising dialyzing said extra fiber space cell supernatant against tissue culture medium to produce extra fiber space conditioned medium.

14. The method of claim 1, wherein said the genomes of said lymphoid cells have been modified by the insertion of cloned DNA that encodes a protein that is expressed when said cells are used in adoptive immunotherapy.

15. The method of claim 14, wherein said cloned DNA encodes at least one protein selected from the group consisting of traceable marker proteins, therapeutically effective proteins, and proteins responsible for drug resistance or sensitivity.

16. A improved method for the growth of <u>in vitro</u> expanded lymphoid cells, wherein said cells are tumor infiltrating lymphocytes, comprising:

(a) suspending cells that are derived from a resected tumor tissue in cell tissue culture medium;

(b) culturing said suspension in the presence of an effective amount at least one cytokine that is capable

of promoting the expansion of tumor infiltrating lymphocytes, wherein said effective amount is an amount sufficient to effect the expansion of the tumor infiltrating lymphocytes in said suspension;

(c) inoculating the extra fiber space of a hollow fiber bioreactor that is a component of a hollow fiber culture system with said cultured suspension of tumor infiltrating lymphocytes;

(d) perfusing said bioreactor with tissue culture medium that contains an effective amount of at least one cytokine that is capable of promoting the expansion of tumor infiltrating lymphocytes, wherein said effective amount is an amount sufficient to effect said specific expansion and said tissue culture medium sustains the cell division and growth of said tumor infiltrating lymphocytes; and

(e) culturing said tumor infiltrating lymphocytes in said bioreactor in the presence of said tissue culture medium for a time sufficient to obtain a therapeutically effective number of said tumor infiltrating lymphocytes, wherein said therapeutically effective number is the number of said cells that is at least sufficient to achieve a therapeutic effect when said cells are used in adoptive immunotherapy.

17. The method of claim 16, wherein said cytokine is at least one cytokine selected from the group consisting of interleukin-1, interleukin-2, interleukin-4, and interleukin-6

18. The method of claim 16, further comprising harvesting the contents of the extra fiber space of the bioreactor, wherein said contents include said tumor infiltrating lymphocytes and the extra fiber supernatant.

19. The method of claim 18, further comprising re-instituting perfusion of said bioreactor and culturing the residual tumor infiltrating lymphocytes that remain in the extra fiber space of said bioreator after said first harvest for a time sufficient to obtain a therapeutically effective amount of said tumor infiltrating lymphocytes; wherein said perfusion and harvesting are re-instituted at least once.

20. The method of claim 18, wherein said medium also contains an effective amount of at least one tumor specific target antigen, wherein said effective amount is at least sufficient to select for the expansion of at least one subpopulation of tumor infiltrating lymphocytes that specifically recognize said antigen.

21. The method of claim 20, wherein said target antigen is only introduced into the medium in the extra fiber space of said bioreactor.

22. The method of claim 18, further comprising pelleting and removing the cells from said contents of the extra fiber space to produce an extra fiber space cell supernatant.

23. The method of claim 22, further comprising dialyzing said extra fiber space cell supernatant against tissue culture medium to produce extra fiber space conditioned medium.

24. The method of claim 16, wherein the genomes of said tumor infiltrating lymphocytes have been modified by the insertion of cloned DNA that encodes a protein that is expressed when said lymphocytes are used in adoptive immunotherapy.

25. The method of claim 24, wherein said cloned DNA encodes at least one protein selected from the

group consisting of traceable marker proteins, therapeutically effective proteins, and proteins responsible for drug resistance or sensitivity.

26. A hollow fiber bioreactor, comprising:

(a) a hollow shell that is suitable for the growth of mammalian cells,

(b) a plurality of hollow fibers encased within said shell, wherein said fibers are semi-permeable and suitable for the growth of mammalian cells on or near them;

(c) lymphoid cells, which have been cultured in the presence of an effective amount of at least one growth promoting substance that specifically promotes the expansion of at least one subpopulation of said cells, which can be used in adoptive immunotherapy, on or near said fibers; and

(d) tissue culture medium within said shell and surrounding said fibers that contains an effective amount of at least one growth promoting substance that specifically expands a therapeutically useful subpopulation of said lymphoid cells, wherein said effective amount is an amount sufficient to effect said specific expansion, said tissue culture medium sustains the cell division and growth of said subpopulation, and said therapeutic use is adoptive immunotherapy;

27. The bioreactor of claim 26, wherein said growth promoting substance is at least one substance selected from the group consisting of mitogens and cytokines.

28. The bioreactor of claim 27, wherein said cytokine is at least one cytokine selected from the groups of cytokines consisting of interleukin-1,

interleukin-2, interleukin 4, and interleukin 6.

29. The bioreactor of claim 26, wherein said lymphoid cells are tumor infiltrating lymphocytes.

30. In vitro expanded lymphoid cells produced by the method of claim 1.

31. In vitro expanded lymphoid cells produced by the method of claim 6.

32. In vitro expanded lymphoid cells produced by the method of claim 14.

33. Tumor infiltrating lymphocytes produced by the method of claim 16.

34. Tumor infiltrating lymphocytes produced by the method of claim 19.

35. Tumor infiltrating lymphocytes produced by the method of claim 24.

36. The extra fiber space cell supernatant produced by the method of claim 12.

37. Extra fiber space conditioned medium produced by the method of claim 13.

38. The extra fiber space cell supernatant produced by the method of claim 22.

39. Extra fiber space conditioned medium produced by the method of claim 23.

40. An improved method for producing in vitro expanded lymphoid cells, comprising culturing said cells in the presence of an effective amount of extra fiber space conditioned medium, wherein said amount is effective to stimulate the rate of growth of said expanded cells at least about 50% more than the growth of said cells in its absence.

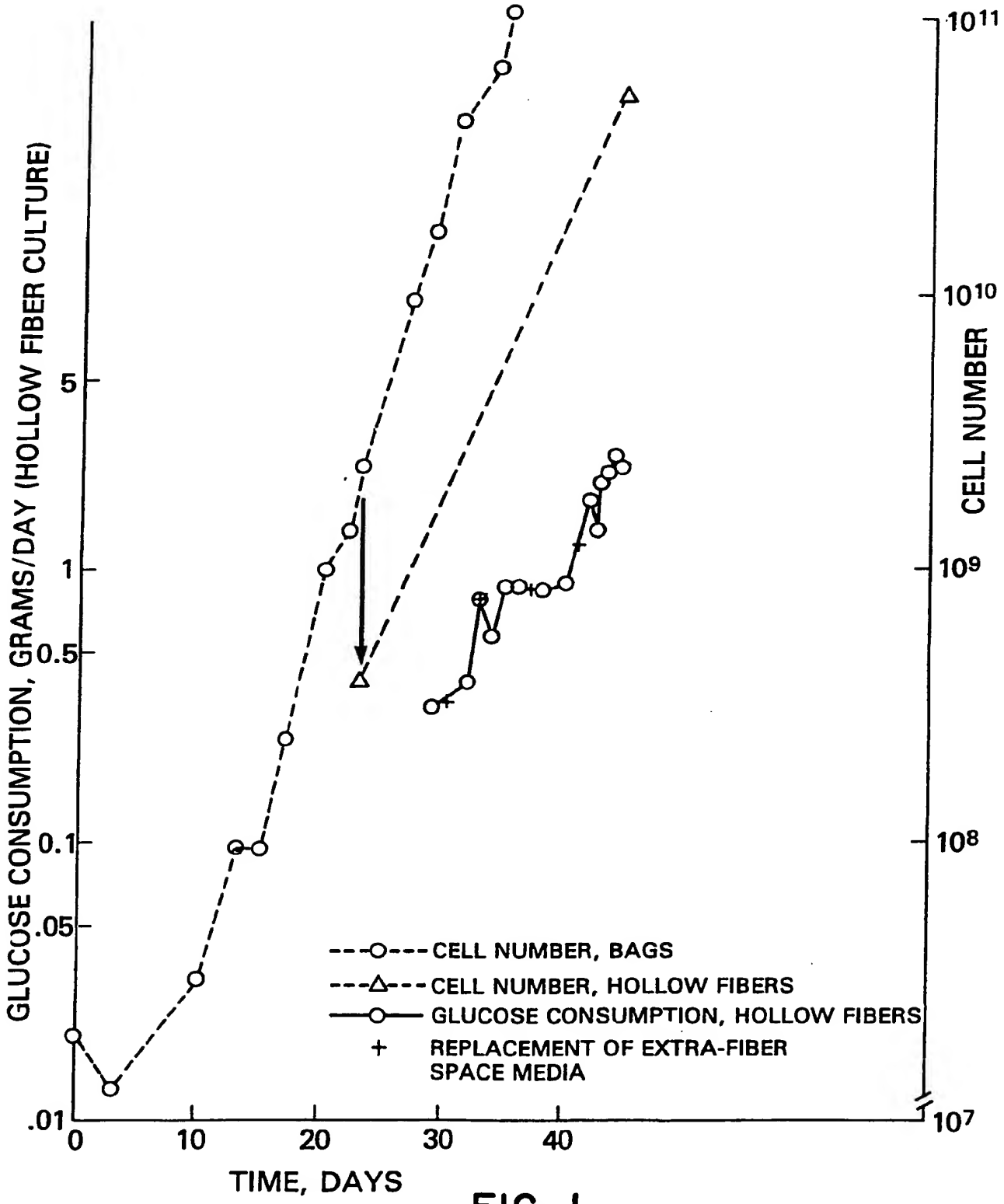
GLUCOSE CONSUMPTION, GRAMS/DAY (HOLLOW FIBER CULTURE)
5
1
0.5
0.1
.05
.01
0
10
20
30
40
TIME, DAYS
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
CELL NUMBER
CELL NUMBER, BAGS
CELL NUMBER, HOLLOW FIBERS
GLUCOSE CONSUMPTION, HOLLOW FIBERS
REPLACEMENT OF EXTRA-FIBER SPACE MEDIA

FIG. 1

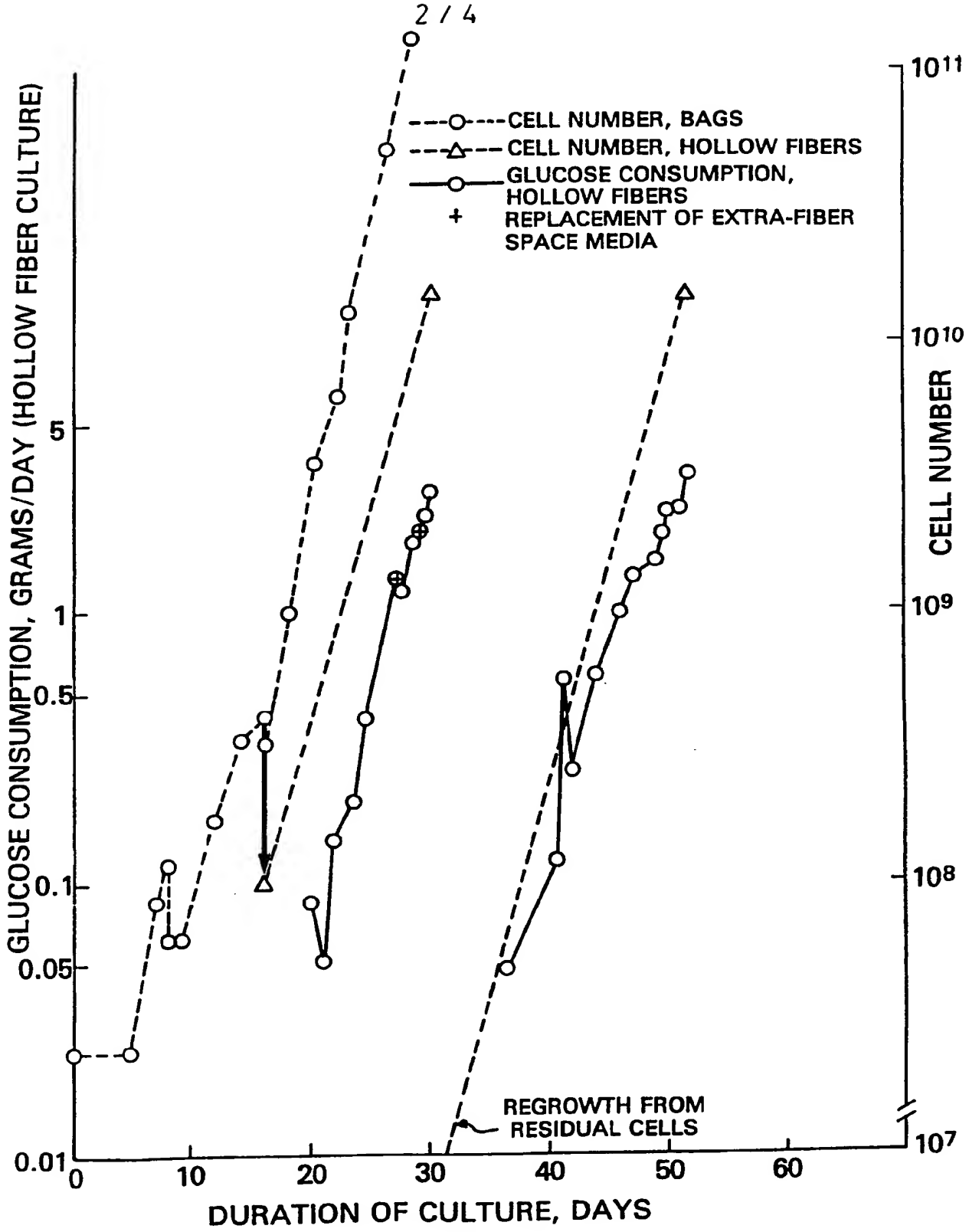
CELL NUMBER, BAGS
CELL NUMBER, HOLLOW FIBERS
GLUCOSE CONSUMPTION, HOLLOW FIBERS
REPLACEMENT OF EXTRA-FIBER SPACE MEDIA
GLUCOSE CONSUMPTION, GRAMS/DAY (HOLLOW FIBER CULTURE)
CELL NUMBER
DURATION OF CULTURE, DAYS
REGROWTH FROM RESIDUAL CELLS
0.01
0.05
0.1
0.5
1
5
0
10
20
30
40
50
60
10^7
10^8
10^9
10^{10}
10^{11}

FIG. 2

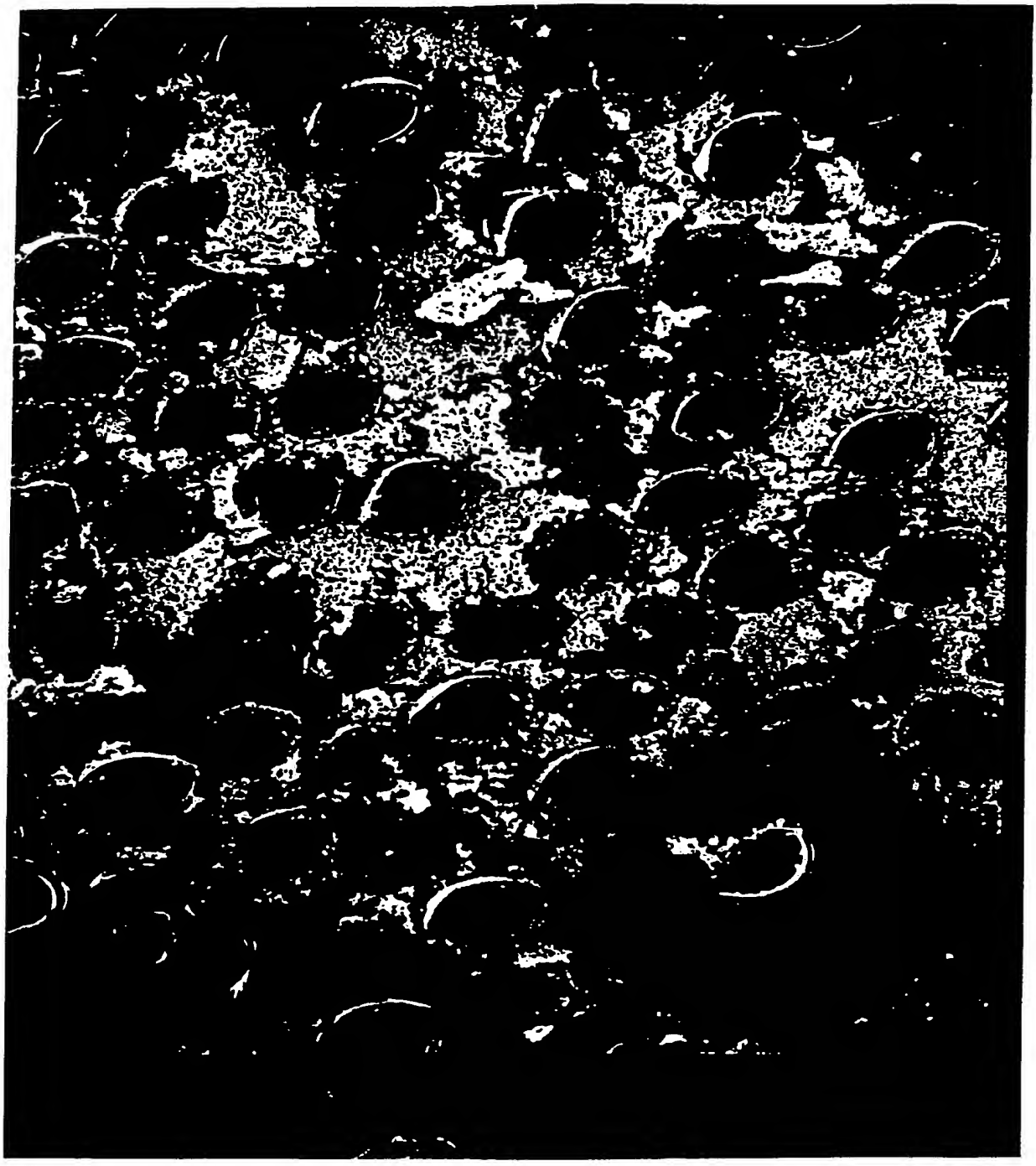

FIG. 3A

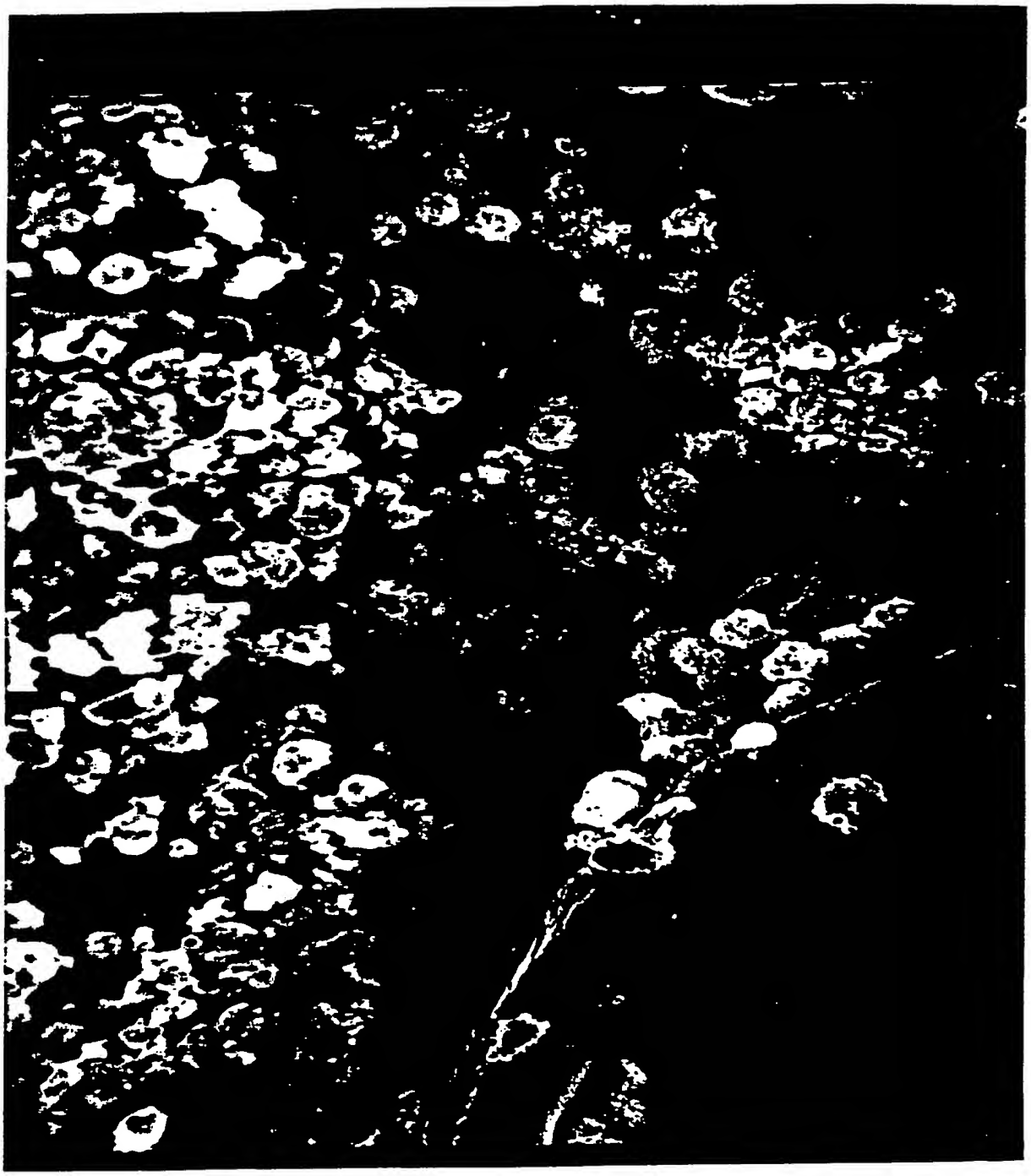

FIG. 3B

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/05051

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 5/00,5/08,5/02; C12M 3/04,3/06

U.S.Cl.: 435/240.242,240.21,240.3,285

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.Cl.	435/240.242,240.21,240.3,285

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y X	US, A, 3,883,393 (KNAZEK et al.) 13 May 1975, see entire document.	1-25,30-40 26-29
Y	US, A, 4,391,912 (YOSHIDA et al.) 05 July 1983, see figures 2-7, examples 3-5.	1-40
Y	US, A, 4,690,915 (ROSENBERG) 01 September 1987, see column 4, lines 8-48; see column 3-column 4 bridge paragraph.	1-40
Y	Journal of Immunological Methods Vol. 88, 1986, Muul, et al., "Large Scale Production Of Human Lymphokine Activated Killer Cells For Use In Adoptive Immunotherapy", pages 265-275 (see entire document).	1-17,30,31, 36,37,40

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of This International Search Report [2]
17 December 1990	22 JAN 1991
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA/US	George C. Elliott

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Cancer Research, Vol. 49, No. 7, Published 01 April 1989, Sawamura et al. "Antitumor Activity And Surface Phenotypes Of Human Glidma-Infiltrating Lymphocytes After In-Vitro Expansion In The Presence Of Interleukin-2" pages 1843-1849 (see abstract).	1-25,30-40

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-35, drawn to a method of producing activated lymphocytes, the lymphocytes produced by the method and an apparatus for carrying out the method, classified

(See attachment).

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

Telephone practice

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment To PCT/ISA/210
Continuation of Part VI, OBSERVATIONS

in Class, 435, subclasses 240.21, 240.2 and 285.

II. Claims 36-39, drawn to conditioned medium, classified in Class 435, subclass 240.3, for example.

III. Claim 40, drawn to a method of culturing cells in conditioned medium, classified in Class 435, subclass 240.21, for example.